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TIR Domain-Containing Adapter-Inducing Beta Interferon (TRIF) Mediates Immunological Memory against Bacterial Pathogens

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Induction of adaptive immunity leads to the establishment of immunological memory; however, how innate immunity regulates memory T cell function remains obscure. Here we show a previously undefined mechanism in which innate and adaptive immunity are linked by TIR domain-containing adapter-inducing beta interferon (TRIF) during establishment and reactivation of memory T cells against Gram-negative enteropathogens. Absence of TRIF in macrophages (Mϕs) but not dendritic cells led to a predominant generation of $CD4^+$ central memory T cells that express IL-17 during enteric bacterial infection in mice. TRIF-dependent type I interferon (IFN) signaling in T cells was essential to Th1 lineage differentiation and reactivation of memory T cells. TRIF activated memory T cells to facilitate local neutrophil influx and enhance bacterial elimination. These results highlight the importance of TRIF as a mediator of the innate and adaptive immune interactions in achieving the protective properties of memory immunity against Gram-negative bacteria and suggest TRIF as a potential therapeutic target.

Host defense against bacterial pathogens utilizes innate phagocytes and $CD4^+$ T cells, and successful interaction between innate and adaptive immunity establishes immunological memory. A fine interplay between innate and adaptive immune responses is necessary to eliminate pathogenic bacteria from the gastrointestinal tract without destruction of normal flora, mucosal barrier function, and gut homeostasis. However, the mechanisms regulating the interactions between innate and adaptive immunity during enteric bacterial infections have yet to be fully determined.

Innate immunity covers immediate host defense against pathogens in a non-antigen-specific manner while the body is conducting initiation and calibration of adaptive immunity. In this system, pathogen-experienced antigen-presenting cells (APCs) induce differentiation of cytotoxic and helper T (Th) cells that form pathogen-specific acquired immunity. Multiple types of Th cells are generated in local lymphoid tissues during infection, while Th17 cell generation is dominant in the intestine (1). The antibacterial properties of Th17 cells have been observed in lung infections with Gram-negative extracellular bacteria (2, 3). In the intestine, however, the role of Th17 cells in host resistance to bacterial infection seems to be more complicated, as they may work as innate immune cells (4, 5). Although the importance of memory $CD4^+$ T cells in host defense against bacterial infection has been well established, the exact extent of coverage by memory Th17 cells has yet to be determined.

TIR domain-containing adapter-inducing beta interferon (TRIF) is an adapter molecule that transduces intracellular signaling upon recognition of Gram-negative bacteria by Toll-like receptor 4 (TLR4) or double-stranded-RNA (dsRNA) viruses by TLR3 (6). Our previous findings regarding the unique role of innate TRIF signaling in intestinal defense against Gram-negative bacteria along with the evidence that TRIF is required for induction of costimulatory molecules and major histocompatibility complex (MHC) class II antigens suggest that TRIF may play an important role at the innate and adaptive interface (7–9).

In this study, we sought to determine the role of TRIF signaling in establishing immunological memory as well as in conferring protective immunity against Gram-negative bacterial infection. We show that TRIF-deficient ($Trif^{LPS2}$) mice failed to demonstrate increased resistance to secondary infection. TRIF deficiency resulted in the enhanced generation and maintenance of $CD4^+$ central memory T (T_{CM}) cells that expressed interleukin 17 (IL-17) in an antigen-specific manner. These IL-17^+ $CD4^+$ T cells facilitated neutrophil influx to the primary infection site and conferred on macrophages (Mϕs) full bactericidal function to eliminate Gram-negative pathogens only when TRIF signaling was present in innate immune cells. Therefore, our results highlight the importance of TRIF in regulating the balance between innate and adaptive immune responses to develop immune resistance to reinfection and suggest its potential as a novel therapeutic target or as a preventative vaccine candidate.

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MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6J, Trif^{LPS2}, and OT-II mice and mice expressing beta interferon with yellow fluorescent protein (IFN-β–YFP) and IL-17 with green fluorescent protein (IL-17-GFP) were purchased from Jackson Laboratory, and Stat1$^{-/-}$ mice were from Taconic Biosciences. IFN-γ Thy1.1 mice and RAG-OT-II mice were gifts from Casey T. Weaver (University of Alabama) and from George Liu (Cedars-Sinai Medical Center [CSMC]), respectively. IL-17–IFN-γ double reporter mice were generated by crossing IL-17–GFP mice and IFN-γ–Thy1.1 mice. Mice were bred and housed under specific-pathogen-free conditions. F_2 littermates were genotyped by TransnetYX and used for infection experiments. All protocols were approved by the CSMC Institutional Animal Care and Use Committee.

Cell preparation and purification. Single-cell suspensions from the spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PP) were prepared by mechanical disruption with 70-μm nylon mesh. Peritoneal Mϕs were isolated as described previously (7). Exclusion of floating cells after 48 h incubation of peritoneal lavage allowed us to collect macrophages (over 97% of adherent cells expressed F4/80). WT naive CD4$^+$ T cells from the spleen or the MLN were purified by magnetic sorting using the CD4$^+$ T cell isolation kit with CD62L microbeads (Miltenyi Biotec). Dendritic cells were prepared from the MLN using CD11c (N418) microbeads (Miltenyi Biotec).

***Yersinia enterocolitica* infection.** Eight- to 12-week-old male mice of each genotype were orogastrically inoculated with *Y. enterocolitica* (WA-314 serotype O:8) using a 22-gauge, round-tipped feeding needle (Fine Science Tools) (10). Mice were given 1×10^7 CFU of *Y. enterocolitica* for the primary infection. To study immunological memory, mice received 1×10^5 CFU *Y. enterocolitica* followed by reinfection (5×10^7 CFU) in 4 weeks. The blocking anti-IFNAR1 antibody or its isotype control was administered the day before reinfection and at days 2 and 4 after reinfection (100 μg intraperitoneally [i.p.]/mouse; Leinco Technologies). Recombinant IFN-ζ was administered daily for the first 3 days of reinfection (30,000 U i.p./mouse, Leinco Technologies).

Mϕ immunization model. Peritoneal Mϕs isolated from WT and Trif^{LPS2} mice were incubated with *Y. enterocolitica* lysate (100 μg/ml) for 60 min at 37°C. Cells were washed with phosphate-buffered saline (PBS) and injected into WT or Trif^{LPS2} mice (5×10^5 cells i.p./mouse). Immunization was performed twice at 2-week intervals.

***Y. enterocolitica*-specific CD4$^+$ memory T cell transfer.** WT and Trif^{LPS2} mice were orogastrically infected with *Y. enterocolitica* (1×10^5 CFU). CD4$^+$ T cells were purified from the spleen and the MLN 4 weeks postinfection and were injected into WT or Trif^{LPS2} mice (5×10^6 cells i.p./mouse). Mice were infected the next day with 5×10^7 CFU *Y. enterocolitica*.

***In vitro* T cell differentiation.** Splenic naive CD4$^+$ T cells from WT or OT-II mice were cocultured with peritoneal Mϕs or MLN dendritic cells (DCs) from WT and Trif^{LPS2} mice (5:1 ratio) in the presence of ovalbumin (10 μg/ml) plus lipopolysaccharide (LPS) (5 ng/ml), *Y. enterocolitica*, *Salmonella enterica* serovar Typhimurium, or *Escherichia coli* lysates (100 μg/ml) for 72 h. CD4$^+$ T cells were stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS for 5 min at 37°C. Anti-IFNAR1 antibody (20 μg/ml) and IFN-ζ (30 ng/ml) (Leinco Technologies) were used to block and induce type 1 IFN signaling, respectively. After 72 h of incubation, differentiated T cells were analyzed by fluorescence-activated cell sorting (FACS) or subjected to Western blot analysis.

Western blot analysis. Protein (30 μg) from T cell lysate was subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking with 5% milk containing 2% bovine serum albumin, the membrane was blotted with antibodies against RORγt (ab78007), T-bet (ab91109), pSTAT1 (ab29045), and STAT1 (ab92509), all purchased from Abcam, and pSTAT3 (9145S), STAT3 (9132), and β-actin (4967S), all from Cell Signaling Technology, followed by suitable secondary antibody conjugated with horseradish peroxidase (HRP). The membrane was exposed on radiographic film using the SuperSignal West Dura kit (Thermo Scientific). Band intensity was calculated using NIH Image 1.62.

RNA sequencing. WT and Trif^{LPS2} peritoneal Mϕs were treated with *Y. enterocolitica* lysate (100 μg/ml) for 24 h. Three independent samples from each condition were pooled to extract total RNA using TRIzol (Ambion). RNA library preparations, sequencing, and initial bioinformatics analysis were conducted at Genewiz, LLC, by using a NEBNext Ultra RNA library prep kit, Illumina HiSeq 2500, and the HiSeq control software. The sequencing libraries were multiplexed and clustered using the cBOT from Illumina. Raw sequence data (.bcl files) were converted to fastq files and demultiplexed using the Illumina CASSAVA 1.8.2 program.

Mϕ bactericidal assay. A bactericidal assay was performed using a previously described method with modifications (11). Pathogen-specific CD4$^+$ T cells expressing IFN-γ or IL-17 were sorted using an AriaIII FACS (Becton Dickinson). Mϕs were incubated with CD4$^+$ T cells (1:1 ratio; 1×10^5 cells/well) expressing IFN-γ or IL-17. Recombinant IL-17 (3 ng/ml) (eBioscience) was added for 60 min at 37°C. Peritoneal Mϕs from WT and Trif^{LPS2} (1×10^5 cells/well) were infected with *Y. enterocolitica*, *S. enterica* serovar Typhimurium, or *E. coli* (multiplicity of infection [MOI], 50) for 30 min in 96-well plates. Cells were further incubated for 6 h in the presence of gentamicin (20 μg/ml). Supernatants were removed, and Mϕs were lysed with 200 μl distilled water. Lysed Mϕs were plated on *Yersinia*-specific agar for *Y. enterocolitica* and LB agar for *S. enterica* serovar Typhimurium and *E. coli*. Data were expressed as CFU per milliliter.

Real-time PCR. One microgram of RNA was used as the template for single-strand-cDNA synthesis using the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed for IFN-γ, IL-17, Spp1, Cxcl10, IFN-ζ, IL-18, IL-27, Lif, IL-15, Cxcl1, Cxcl2, Cxcl5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and β-actin (see Table S1 in the supplemental material). The cDNA was amplified using Maxima SYBR green/ROX (carboxy-X-rhodamine) (Thermo Scientific) on a Realplex Mastercycler (Eppendorf). Relative expression levels were calculated with the comparative $2^{-\Delta\Delta CT}$ method using β-actin as the endogenous control.

Cell staining and FACS analysis. Surface staining of CD3, CD4, CD44, CD62L, CD127, and Gr1 was performed according to the manufacturer's instructions (eBioscience, San Diego, CA). Intracellular staining of IFN-γ, IL-17, IL-4, and RORγt was performed after incubation with phorbol myristate acetate (PMA) (50 ng/ml), ionomycin (350 ng/ml), and GogiPlug (0.8 μl/ml) for 4 h. FACS analyses were performed on an LSRII flow cytometer with FACS Diva (BD) and FlowJo (Tree Star).

ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were carried out per the manufacturer's protocol (R&D Systems). For antigen presentation, CD11c$^+$ DCs were purified from the spleen by using CD11c (N418) microbeads (Miltenyi Biotec) and incubated with *Y. enterocolitica* lysate (100 μg/ml) for 12 h. CD4$^+$ T (1×10^5 cells/well) cells purified from the MLN were added to each well and were cocultured with CD11c$^+$ DCs (2×10^4 cells/well) for 18 h. Spots were developed with AEC (3-amino-9-ethylcarbazole) substrate (BD Bioscience) according to the manufacturer's instructions. The membrane was read by an automated reader (CTL Immunospot) for quantitative analysis of the number of spots per 1×10^5 cells plated, and subtracted background values were defined by CD11c cells alone.

Statistical analysis. A Kaplan-Meier survival curve was generated for infected mice, and statistical differences were analyzed by chi-square test. Student's *t* test was used for 2 independent groups of samples. One-way analysis of variance (ANOVA) was used for more than 2 independent groups of samples, followed by Tukey's multiple comparison tests. Fisher's exact test was used to determine probability. All tests were performed with GraphPad Prism (Version 5.0b), and a P value of <0.05 was considered statistically significant.

RESULTS

Mϕ-mediated Th1 cell differentiation in response to Gram-negative bacteria requires TRIF. Most bacterial pathogens that

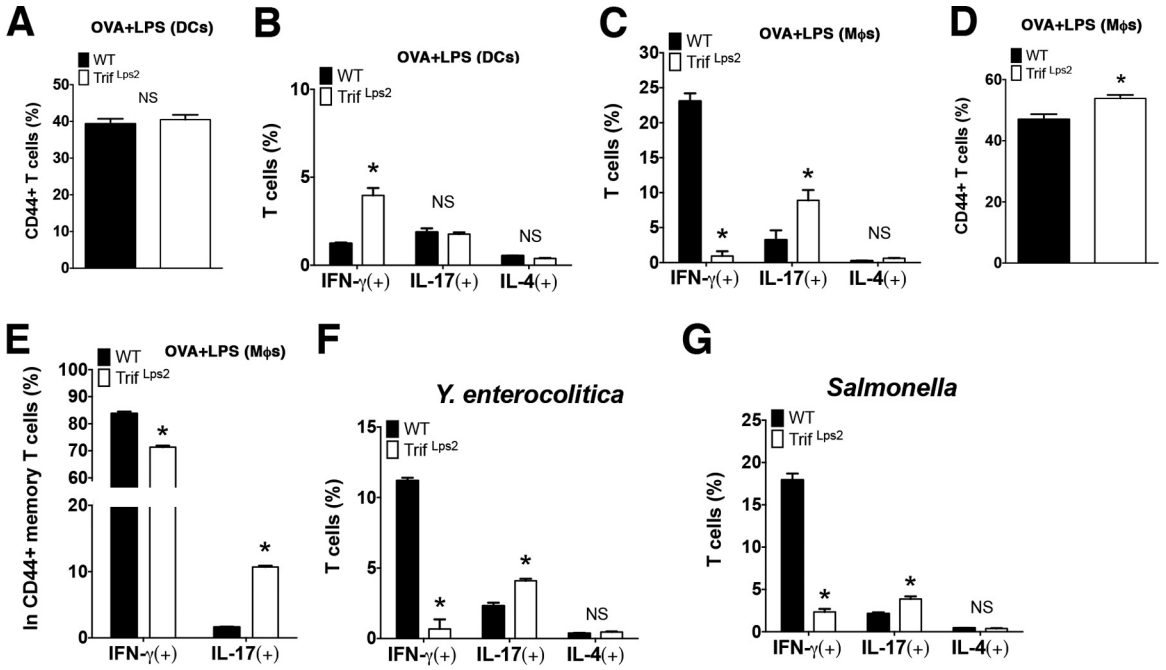

FIG 1 TRIF signaling is responsible for Mϕ-mediated Th1 cell differentiation. (A and B) Percent generation of $CD44^+$ and lineage specific $CD4^+$ T cells after coculture with DCs in the presence of OVA plus LPS. (C) Th cell lineages in $CD4^+$ T cells differentiated with Mϕs in the presence of OVA plus LPS. (D) $CD44^+$ expression in $CD4^+$ T cells. (E) Th cell lineages in $CD44^+$ $CD4^+$ T cells differentiated with Mϕs in the presence of OVA plus LPS. (F) Th cell lineages in $CD4^+$ T cells generated with Mϕs in the presence of *Y. enterocolitica* lysate. (G) Th cell lineages generated with Mϕs in the presence of *S. enterica* serovar Typhimurium lysate. Values are averages from 3 independent experiments ($n = 9$). Error bars show standard errors of the means (SEM). *, $P < 0.05$; NS, not significant.

cause serious gastrointestinal infection are Gram-negative species. To determine the role of TRIF signaling in inducing antigen-specific Th cells against Gram-negative bacteria, naive OT-II cells were cocultured with either DCs from the mesenteric lymph nodes (MLN) or peritoneal Mϕs isolated from WT and $Trif^{LPS2}$ mice in the presence of ovalbumin (OVA) and LPS. After 3 days, the expression of the memory marker on T cells was similar between cocultures with WT DCs and $Trif^{LPS2}$ DCs (Fig. 1A). The expression of lineage-associated cytokines from T cells was also similar between cocultures with WT DCs and $Trif^{LPS2}$ DCs, except that $Trif^{LPS2}$ DCs had greater differentiation of IFN-γ^+ Th1 cells than WT DCs (Fig. 1B). In contrast, $Trif^{LPS2}$ Mϕs differentiated fewer Th1 cells and more Th17 cells than WT Mϕs (Fig. 1C). Almost no Th2 cell differentiation was found in both DC and Mϕ coculture settings (<1%). Although slightly more T cells expressed the memory marker when cocultured with $Trif^{LPS2}$ Mϕs than with WT Mϕs, similar alterations in the lineage populations were found among these memory cells induced by $Trif^{LPS2}$ Mϕs (Fig. 1D and E). This pattern of Th cell differentiation was also observed when T cells were differentiated with WT or $Trif^{LPS2}$ Mϕs in response to *Y. enterocolitica* (Fig. 1F) or *S. enterica* serovar Typhimurium lysates (Fig. 1G). These results suggest that Mϕ-dependent induction of antigen-specific Th1 cell differentiation and the regulation of Th17 cell differentiation in response to Gram-negative bacteria require TRIF signaling.

Memory T cells skew toward a Th17 response and impaired protective immunity in the absence of TRIF. We next sought to determine whether the development and function of $CD4^+$ memory T cells were affected by TRIF signaling *in vivo*. WT and $Trif^{LPS2}$ mice were infected first with *Y. enterocolitica* (1×10^5 CFU), followed by reinfection (5×10^7 CFU) in 28 days. WT mice significantly improved survival during reinfection, indicating establishment of effective immunological memory (Fig. 2A). However, the improved resistance to reinfection was not observed in $Trif^{LPS2}$ mice (Fig. 2B), and their mortality rate was significantly higher than that of WT mice (not shown in the same graph).

The partial resistance seen in the $Trif^{LPS2}$ mice led us to compare the proportion of memory cells in the MLN between WT and $Trif^{LPS2}$ mice 7 days after reinfection. Flow cytometry (FCM) revealed almost equal generation of $CD4^+$ $CD44^+$ memory cells but an increased proportion of T_{CM} cells in the MLNs of $Trif^{LPS2}$ mice compared to WT mice (Fig. 2C). Consistent with our *in vitro* data, more $CD4^+$ $CD44^+$ memory T cells in $Trif^{LPS2}$ mice expressed IL-17 than WT memory T cells in the MLN (Fig. 2D). In contrast, the number of IFN-γ^+ memory T cells in the MLN was less in $Trif^{LPS2}$ mice than WT mice (Fig. 2D). Because $Trif^{LPS2}$ mice showed higher mortality than WT mice despite greater generation of T_{CM} cells, we assessed bacterial dissemination to measure the protective potential of these memory T cells and found that 50% of WT mice and 88.9% of $Trif^{LPS2}$ mice had splenic dissemination of *Y. enterocolitica* 7 days after secondary infection ($P < 0.05$). In addition, the abundance of *Y. enterocolitica* in the spleen was significantly higher in $Trif^{LPS2}$ mice than WT mice (Fig. 2E). These results suggest that T_{CM} cell generation and Th17 lineage differentiation are regulated by TRIF, which result in enhanced Th1 response and thus may be required for effective memory immunity.

Effective memory T cell immunity is conferred by TRIF signaling in non-T cell compartments. To understand why increased generation of IL-17^+ memory cells did not promote resis-

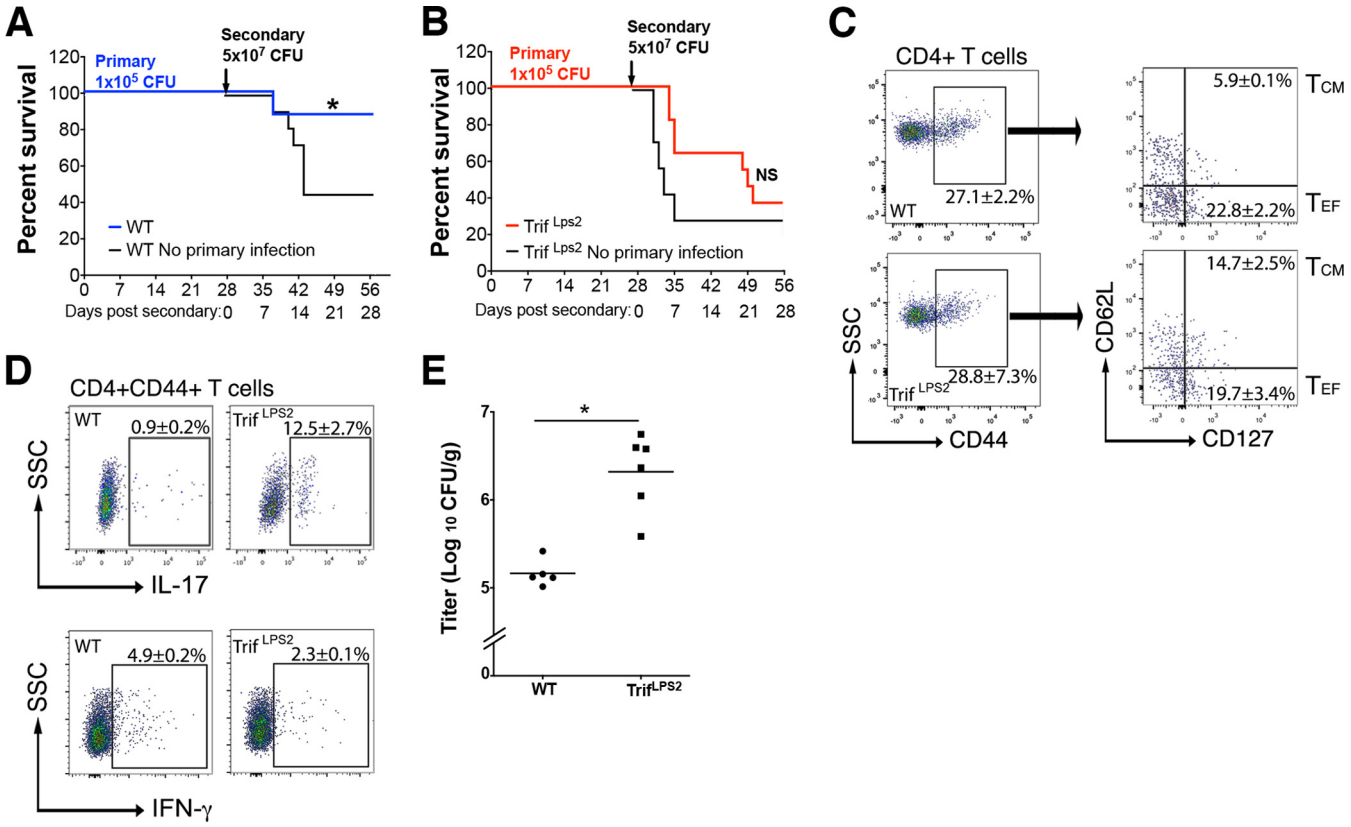

FIG 2 TRIF mediates protective immunity by regulating memory CD4$^+$ T cell differentiation. (A) Percent survival of WT mice in reinfection (primary, 1×10^5 CFU; secondary, 5×10^7 CFU *Y. enterocolitica*) with or without primary infection ($n = 10$ each; *, $P < 0.05$). (B) Survival of Trif^{LPS2} mice with or without primary infection ($n = 10$ each; NS, not significant). (C) FCM analysis of CD44$^+$ CD4$^+$ T cells isolated from the MLN 7 days after reinfection. Results are representative of 3 independent experiments and are means ± SEM ($n = 6$ each). (D) FCM analysis of IFN-γ and IL-17 in CD44$^+$ T cells isolated from the MLN 7 days after reinfection. Results are representative of 3 independent experiments and are means ± SEM ($n = 6$ each). (E) *Y. enterocolitica* colonization in the spleen 7 days after reinfection (*, $P < 0.05$).

tance to reinfection in Trif^{LPS2} mice, WT mice were infected with *Y. enterocolitica* (5×10^7 CFU) after receiving primed CD4$^+$ T cells isolated from WT and Trif^{LPS2} mice that had been preexposed to *Y. enterocolitica* (1×10^5 CFU, day 28). Consistent with previous reports, increased resistance to *Y. enterocolitica* infection was observed in WT mice by transferring primed WT CD4$^+$ T cells (Fig. 3A) (12). Similar improvement in mortality resulted from transferring primed CD4$^+$ T cells from Trif^{LPS2} mice (Fig. 3A). However, no survival improvement was observed in Trif^{LPS2} mice receiving WT primed CD4$^+$ T cells (Fig. 3A). We confirmed that transferred memory T cells had been equally distributed to the MLN in WT and Trif^{LPS2} mice by using carboxyfluorescein succinimidyl ester (CFSE) labeling. After 7 days of infection, the percentage of CFSE$^+$ cells in the MLN was approximately 4% in both WT and Trif^{LPS2} mice (Fig. 3B). The majority (>75%) of CFSE$^+$ cells were memory cells, as they expressed high levels of CD44 (Fig. 3B). However, within 14 days postinfection, these transferred WT memory T cells began to express more IL-17 and less IFN-γ in Trif^{LPS2} mice than the transferred cells in WT mice (Fig. 3C). In addition, *Y. enterocolitica* dissemination to the spleen was still greater in Trif^{LPS2} mice than WT mice even after the mice received WT memory T cells (Fig. 3D).

Accelerated neutrophil infiltration has been identified as the main antibacterial property of Th17 cells (13, 14). However, we previously showed defective neutrophil infiltration in Trif^{LPS2} mice (7). Increased numbers of Th17 cells in Trif^{LPS2} mice were also found in the Peyer's patches (PP) 4 days after oral infection with *Y. enterocolitica* (Fig. 3E). Therefore, we addressed whether Trif^{LPS2} mice restored the levels of neutrophil infiltration in the PP, the primary invasion site of *Y. enterocolitica*. FCM analysis demonstrated a lower number of Gr1^{hi} cells in the PP of Trif^{LPS2} mice than in WT mice 4 days postinfection (Fig. 3F). Consistently, the expression of chemokines involved in IL-17-mediated neutrophil influx was not higher in the PP of Trif^{LPS2} mice than in WT PP (Fig. 3G). The expression of Cxcl1 and Cxcl2 in response to recombinant IL-17 (rIL-17) was similar between WT and Trif^{LPS2} Mϕs (Fig. 3H). These results indicate that neutrophil recruitment in the PP relies more on TRIF signaling than Th17-dependent chemotaxis.

Next we examined the influence of individual T cell lineages on elimination of bacteria by Mϕs. *Y. enterocolitica*-specific WT Th1 and Th17 cells were generated *in vitro* and individually added to Mϕs undergoing bacterial killing (Fig. 3I). Our results demonstrated that not only Th1 cells but also Th17 cells significantly enhanced bacterial killing in both WT and Trif^{LPS2} Mϕs. However, Trif^{LPS2} Mϕs showed significantly impaired killing of *Y. enterocolitica* compared to WT Mϕs even with the presence of these T cells (Fig. 3H). This difference was not due to an impaired re-

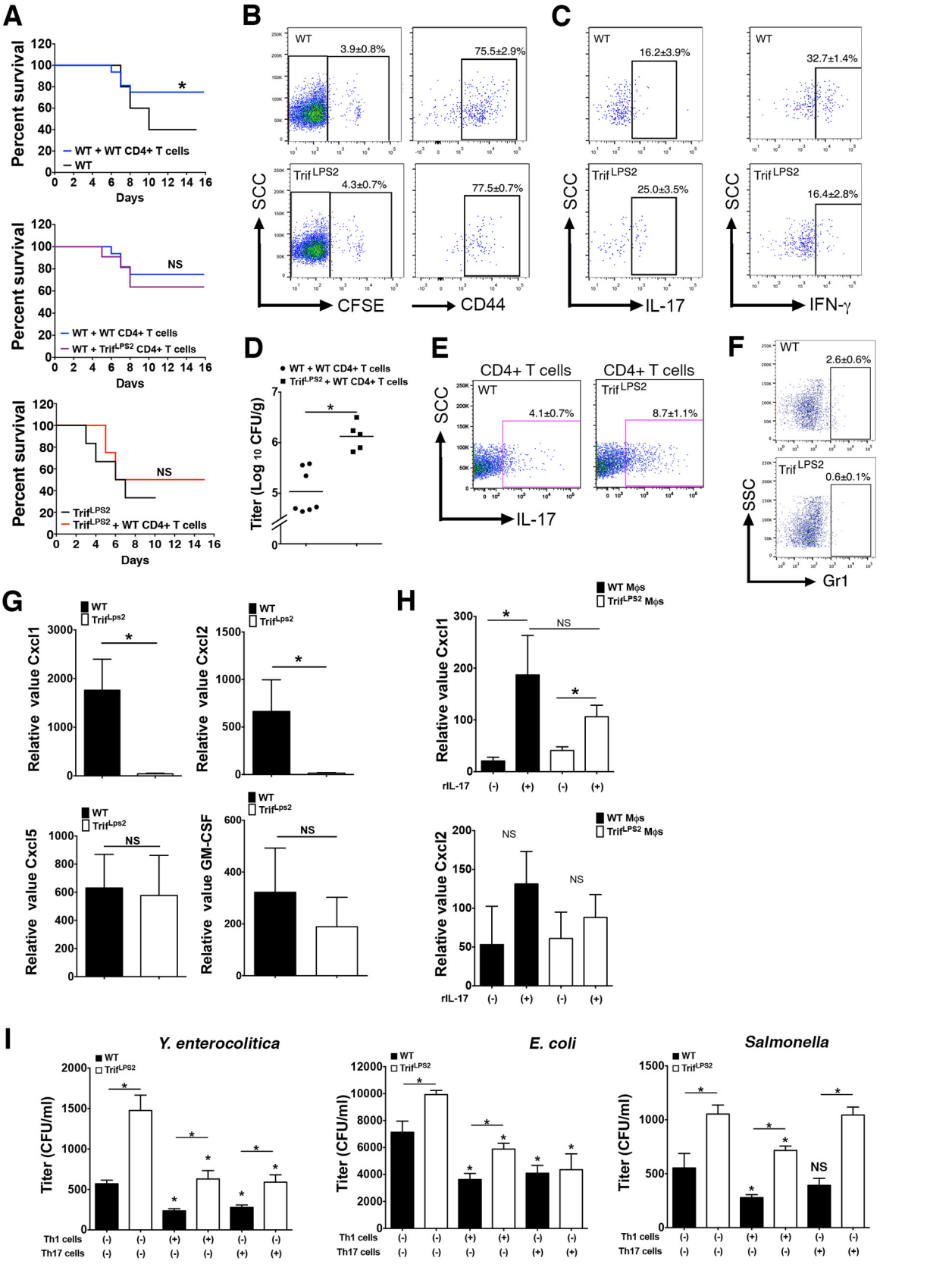

FIG 3 TRIF regulates lineage differentiation and protective immunity in CD4$^+$ memory T cells. (A) Percent survival of WT and Trif^{LPS2} mice given CD4$^+$ memory T cells from preinfected WT or Trif^{LPS2} mice (5×10^7 CFU *Y. enterocolitica*; $n = 7$ each; *, $P < 0.05$). (B) FCM analysis of CFSE-labeled and endogenous memory T cells in the MLN ($n = 4$ each; data are means ± SEM). (C) FCM analysis of IFN-γ and IL-17-expressing memory T cells in CFSE$^+$ cells ($n = 4$ each;

sponse to IL-17 in Trif^{LPS2} Mϕs, as transcriptome sequencing (RNA-seq) data showed similar expression levels of *Il17ra* between WT Mϕs and Trif^{LPS2} Mϕs (data not shown). The influence of Th17 cells in Mϕ bactericidal function seems to be exclusive to extracellular bacteria, because increased bactericidal function by Th17 cells against *Salmonella* was not observed (Fig. 3H). Therefore, induction of protective immunity by memory T cells is dependent upon TRIF signaling in non-T cell compartments.

The robust Th17 cell generation in Trif^{LPS2} mice is antigen specific. To understand when and how Trif^{LPS2} mice generate abundant Th17 cells and whether those Th17 cells are antigen specific, we examined Th17 cell generation during primary *Y. enterocolitica* infection (1×10^7 CFU). Although the number of Th17 cells in the MLN was similar between WT and Trif^{LPS2} mice prior to infection (data not shown), Trif^{LPS2} mice generated almost double the number of Th17 cells in the MLN relative to WT mice 9 days after primary infection (Fig. 4A). In contrast, Th1 cell generation in the MLN was lower in Trif^{LPS2} mice than WT mice. The abundantly generated Th17 cells in Trif^{LPS2} mice were *Y. enterocolitica* specific, as confirmed by ELISPOT assay (Fig. 4B).

To further confirm possible involvement of TRIF signaling in non-T cell compartments in the regulation of Th17 cell generation, we transferred CFSE-labeled WT naive T cells into WT and Trif^{LPS2} mice and infected them with *Y. enterocolitica* (1×10^7 CFU). The expansion of Th17 cells in the CFSE$^+$ population was greater in Trif^{LPS2} recipients than WT recipients 7 days postinfection (Fig. 4C). Therefore, the abundant Th17 cell differentiation in Trif^{LPS2} mice may be regulated by TRIF signaling in APCs from non-T cell compartments.

Th17-biased T cell differentiation is associated with a unique cytokine profile in the absence of TRIF. To test whether the altered generation of Th cells in Trif^{LPS2} mice was due to defective cytokine production in APCs and/or activities of transcription factors in T cells, we examined the cytokine profile expressed by Mϕs after stimulation with *Y. enterocolitica* lysate by RNA-seq. Compared to WT Mϕs, Trif^{LPS2} Mϕs showed 2,186 upregulated and 2,012 downregulated transcripts determined by 2-fold threshold. Among these genes, 35 upregulated and 52 downregulated genes were found to be cytokines or chemokines based on the clustering with David (http://david.abcc.ncifcrf.gov/) and Mayday 2.14 (Fig. 5A). We further found upregulation of *Spp1* and downregulation of *Cxcl10*, *Ifnz*, *IL12b*, *IL-15*, *IL-18*, *IL-27*, and *Lif* in Trif^{LPS2} Mϕs compared to WT Mϕs. The altered expression of these genes has been implicated in Th17 cell polarization (15–17). Consistently, mRNA expression analysis in the MLNs of WT and Trif^{LPS2} mice 9 days after *Y. enterocolitica* infection demonstrated significantly higher expression of *Spp1* and lower expression of *Cxcl10*, *Ifnz*, *IL-27*, and *Lif* in Trif^{LPS2} mice than WT mice (Fig. 5B). These alterations in gene expression in Trif^{LPS2} Mϕs appear to be a universal phenomenon against Gram-negative bacteria, because a similar pattern of these gene expressions was observed in response to *E. coli* lysate (Fig. 5C).

When the activation of transcription factors in naive T cells was assessed after 3 days of coculture with Mϕs in the presence of *Y. enterocolitica* lysate, higher RORγt expression was found in T cells that were cocultured with Trif^{LPS2} Mϕs than T cells cocultured with WT Mϕs (Fig. 5D). In addition, Trif^{LPS2} Mϕs were less able to induce T cell STAT1 activation than WT Mϕs (Fig. 5D). These results indicate that multiple cytokines are positively and negatively involved in abnormal generation of Th17 cells in Trif^{LPS2} mice during Gram-negative bacterial infections, which results in unsuppressed RORγt activation and impaired activation of STAT1 in T cells.

TRIF-dependent regulation of Th17 cell responses involves type I IFN signaling. Type I IFNs are the signature cytokines of TRIF and are potent inducers of STAT1 activation. *Ifnz*, one of the genes downregulated in Trif^{LPS2} Mϕs, encodes a type I IFN and signals through the type I IFN receptor (IFNAR) (18). In addition to *Ifnz*, many of the genes identified by the RNA-seq experiment can be induced by type I IFN signaling (Fig. 5A). Therefore, we addressed whether Th17 cell generation is altered by impaired type I IFN signaling in the absence of TRIF. T cells cocultured with WT Mϕs and *Y. enterocolitica* lysate showed upregulation of RORγt and decreased STAT1 phosphorylation when type I IFN signaling was blocked (Fig. 6A). These results mimic T cells that were cocultured with Trif^{LPS2} Mϕs. When we used STAT1-deficient naive T cells, the generation of RORγt-expressing T cells was increased even after coculture with WT Mϕs (Fig. 6B). Adding back recombinant IFN-ζ during T cell coculture with Trif^{LPS2} Mϕs in turn suppressed expansion of RORγt-expressing T cells (Fig. 6C).

Next we examined the effect of type I IFN signaling in Th17 memory T cell generation in an *in vivo* setting. First we confirmed the cell type producing type I IFNs by infecting IFN-β–YFP reporter mice with *Y. enterocolitica*; 86.5% of IFN-β-expressing cells in PP were F4/80$^+$ cells 48 h postinfection (Fig. 6D). When WT mice were treated with anti-IFNAR1 antibody during the course of secondary *Y. enterocolitica* infection, they demonstrated higher mortality than mice that received control antibody (Fig. 6E). However, adding back recombinant IFN-ζ during the first 3 days of *Y. enterocolitica* reinfection did not show a protective effect in Trif^{LPS2} mice (Fig. 6E). These results suggest that despite the significant requirement of type I IFN signaling for the proper operation of memory immunity, immunological memory established in Trif^{LPS2} mice does not provide resistance to the secondary infection even in the presence of IFN-ζ. Real-time PCR demonstrated significant upregulation of *Spp1* and downregulation of *Cxcl10*, *Ifnz*, *IL-18*, and *IL-27* mRNA in the MLN in WT mice treated with anti-IFNAR1 antibody (Fig. 6F). In addition, blocking type I IFN signaling resulted in increased Th17 cells and, to a lesser extent, decreased Th1 cells in the MLN 7 days after secondary infection (Fig. 6G). Our data suggest that type I IFN signaling is responsible for a large part of TRIF-dependent regulation of

data are means ± SEM). (D) *Y. enterocolitica* colonization in the spleen ($n = 7$ each; *, $P < 0.05$). (E and F) FCM analysis of IL-17$^+$ CD4$^+$ T cells and Gr1^{hi} cells in the PP of WT and Trif^{LPS2} mice ($n = 4$ each; data are means ± SEM). (G) Real-time PCR analysis of chemokine expression in the PPs (4 days postinfection; 1×10^7 CFU *Y. enterocolitica*; $n = 10$ each; *, $P < 0.05$; NS, not significant). (H) Real-time PCR analysis of the mRNA expression of Cxcl1 and Cxcl2 in peritoneal Mϕs 24 h after stimulation with rIL-17 (eBioscience; 1 μg/ml). Data are averages from 2 independent experiments ($n = 14$ each), *, $P < 0.05$; NS, not significant. (I) Killing assay using WT or Trif^{LPS2} peritoneal Mϕs in combination with *in vitro*-generated Th1 and Th17 cells (Th1 and Th17 cells were isolated after coculturing splenic naive T cells from IFN-γ–IL-17 double reporter mice with peritoneal Mϕs in the presence of *Y. enterocolitica* lysate). *Y. enterocolitica*, *E. coli*, or *S. enterica* serovar Typhimurium was used (MOI, 50) ($n = 15$ each; *, $P < 0.05$).

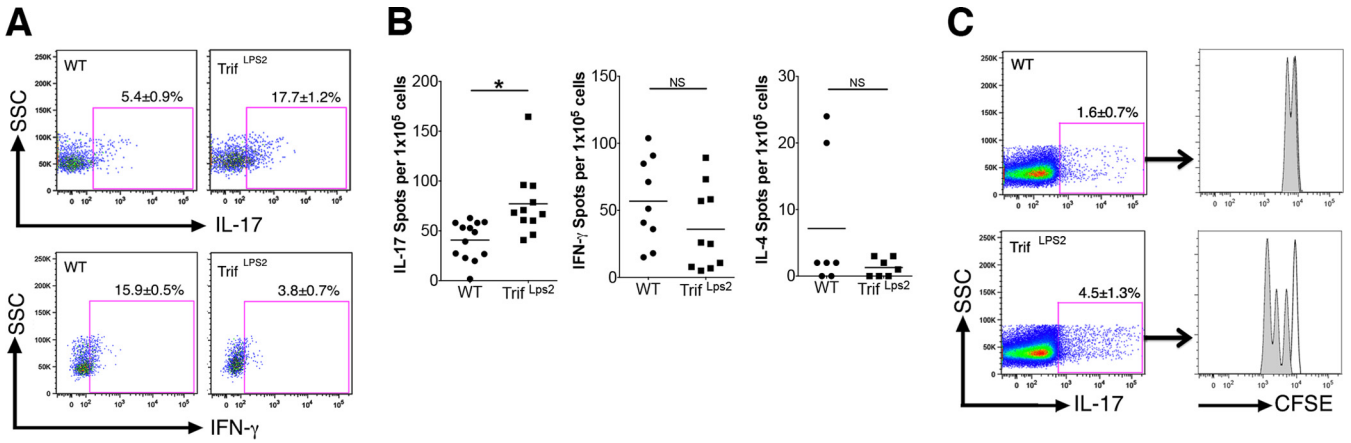

FIG 4 Trif^{LPS2} mice skew Th cell differentiation to Th17 cells during primary infection with *Y. enterocolitica*. *Y. enterocolitica*-specific Th cell expansion in the MLN (9 days postinfection; 1×10^7 CFU). (A) FCM analysis of IFN-γ and IL-17 in CD4$^+$ T cells isolated from the MLN. Data are representative of 2 independent experiments ($n = 4$ each; data are means ± SEM). (B) ELISPOT assay detecting *Y. enterocolitica*-specific CD4$^+$ T cells expressing IFN-γ, IL-17, and IL-4. The graphs show combined data from 3 independent experiments (*, $P < 0.05$; NS, not significant). (C) Mice were injected with CFSE-labeled WT naive CD4$^+$ T cells followed by *Y. enterocolitica* infection (1×10^7 CFU) for 7 days. FCM analysis of proliferating CD4$^+$ T cells in the MLN that express IL-17 was carried out ($n = 4$ each; data are means ± SEM).

Th17 memory T cell generation in response to Gram-negative bacteria.

TRIF-mediated Mϕ elimination of bacteria involves Th cell-mediated memory immunity. To further examine the role of Mϕ TRIF signaling in priming Th17 cells *in vivo*, we employed a Mϕ immunization model. Peritoneal Mϕs from WT and Trif^{LPS2} mice were stimulated with *Y. enterocolitica* lysate and intraperitoneally injected into WT mice, and their potential for priming of Th17 cells in the MLN was compared. About 10% of injected Mϕs were distributed to the MLN within 24 h (data not shown). Trif^{LPS2} Mϕs demonstrated a greater ability to prime Th17 cells but a lesser ability to prime Th1 cells in the MLN than WT Mϕs (Fig. 7A). Consistent with the results from the reinfection experiment, immunization with Trif^{LPS2} Mϕs resulted in more T_{CM} cells in the MLN than WT Mϕ immunization (Fig. 7B). The increased generation of Th17 cells by Trif^{LPS2} Mϕs was *Y. enterocolitica* specific, as determined by ELISPOT assay (Fig. 7C).

In the enteric *Y. enterocolitica* infection model, immunization with WT Mϕs provided strong protective immunity in both WT and Trif^{LPS2} mice, while immunization with Trif^{LPS2} Mϕs did not reduce mortality (Fig. 7D and E). In addition, a significantly higher number of colonies was recovered from the spleens in the survival group of WT mice that were immunized with Trif^{LPS2} Mϕs than from spleens of WT mice immunized with WT Mϕs (Fig. 7F). These results indicate that TRIF signaling in Mϕs regulates Th17 cell differentiation in the MLN, which balances the Th1/Th17 ratio and leads to an appropriate establishment of immunological memory in response to *Y. enterocolitica*.

DISCUSSION

Studies of memory T cells have focused mainly on cytotoxic-T-cell responses against viral pathogens, while relatively little is known about the regulation and generation of CD4$^+$ memory T cells against bacterial pathogens. In addition, how the individual subsets of CD4$^+$ memory T cells contribute to the protective immunity remains unclear. This study sought to examine the mechanisms of the balance between the innate and adaptive immune system, and in particular, the role of TRIF signaling in the regulation of CD4$^+$ memory T cell responses against Gram-negative bacteria. Trif^{LPS2} mice were prone to generate IL-17$^+$ T_{CM} cells in intestinal inductive sites during enteric *Y. enterocolitica* infection, but they could not drive protective immunity upon reinfection. The mechanism underlying the altered memory T cell response in Trif^{LPS2} mice is mainly involved in impaired type I IFN signaling in T cells that are responsible for stabilization and activation of STAT1 during lineage differentiation and reactivation processes. Our results indicate that TRIF-induced chemokines and the IFN signaling in infected Mϕs were indispensable for acquiring protective immunity from memory T cells. These results highlight novel interactions between innate and adaptive immunity that are mediated through TRIF signaling.

During the establishment of immunological memory, activation of innate immunity directs antigen-specific T cell differentiation, which is mediated through cell-cell contact and the sets of cytokines secreted from APCs (19, 20). We found the altered expression of 5 unique genes (*Spp1*, *Cxcl10*, *Ifnz*, *IL-27*, and *Lif*) in Trif^{LPS2} Mϕs that are known to influence Th17 cell differentiation (15–17). All these genes except for *Lif* were regulated by type I IFN signaling, as blocking type I IFN receptor in WT mice during reinfection showed the same alteration of expression of these cytokines in the MLN. Type I IFN-induced STAT1 activation seems to be responsible for suppression of abnormal Th17 cell generation, because activation of STAT1 was impaired in Trif^{LPS2} T cells, and WT Mϕs abundantly generated RORγt-expressing T cells from STAT1$^{-/-}$ naive T cells. *Spp1* and *IL-27* were individually reported to induce STAT1 degradation and activation, respectively (21, 22). In addition, the importance of IL-27 in TRIF-dependent regulation of effector Th17 cell generation in experimental autoimmune encephalomyelitis in mice has been shown (23). Our data are supported by a previous report that showed inhibition of RORγt expression in T cells by STAT1 activation (24). The effective operation of memory immunity also requires type I IFN signaling, as blocking type I IFN signaling in WT mice nullified

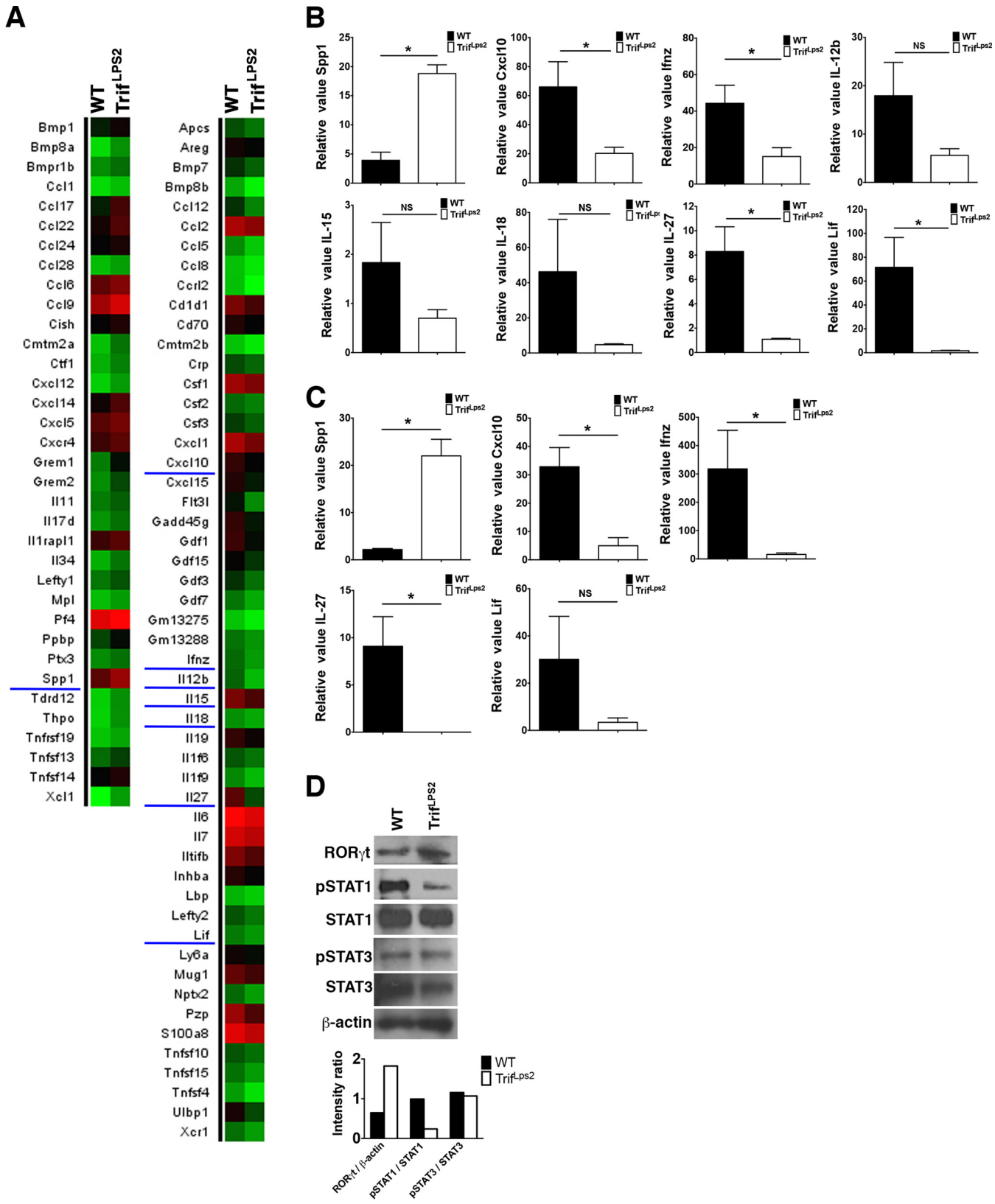

FIG 5 Unique cytokine profile of Trif^{LPS2} Mϕs and altered induction of transcription factors in T cells favor Th17 cell differentiation in response to *Y. enterocolitica*. (A) Heat maps of genes involved in Th cell differentiation determined by RNA-seq using WT and Trif^{LPS2} Mϕs 24 h after incubation with *Y. enterocolitica* lysate. (B) Real-time PCR analysis of gene expression in the MLNs taken from WT and Trif^{LPS2} mice 9 days after *Y. enterocolitica* infection ($n = 10$ each; *, $P < 0.05$; NS, not significant). (C) Real-time PCR analysis of gene expression in WT and Trif^{LPS2} Mϕs 24 h after incubation with *E. coli* lysate ($n = 10$ each; *, $P < 0.05$; NS, not significant). (D) Western blot analysis of transcription factors in WT T cells cocultured with WT and Trif^{LPS2} Mϕs in the presence of *Y. enterocolitica*. Results are representative of 4 independent experiments. The graph shows ratios of the band intensities.

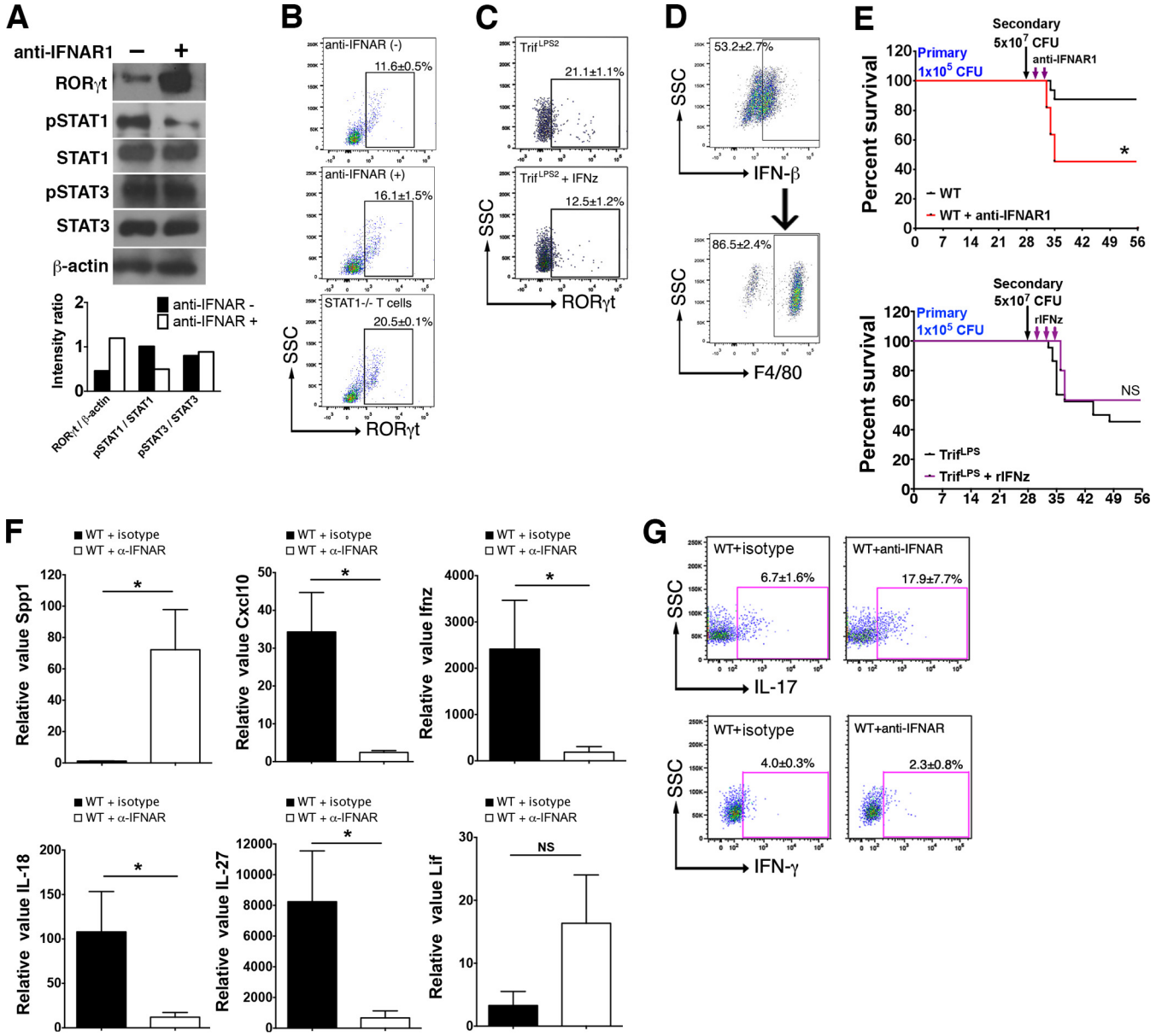

FIG 6 TRIF regulates Th17 cell differentiation by type I IFN signaling. (A) Western blot analysis of transcription factors in WT Th cell differentiation in response to *Y. enterocolitica*, showing the effect of anti-IFNAR1 antibody. Data are representative of 5 independent experiments. The graph shows ratios of the band intensities. (B) FCM analysis of RORγt in WT T cells treated with anti-IFNAR1 antibody, or T cells of STAT1$^{-/-}$ mice during Th cell differentiation in response to *Y. enterocolitica* ($n = 4$ each; data are means ± SEM). (C) The effect of recombinant IFN-ζ on RORγt expression in WT Th cell differentiation while coculturing with Trif^{LPS2} Mϕs and *Y. enterocolitica* lysate ($n = 4$ each; data are means ± SEM). (D) FCM analysis of the F4/80$^+$ component in IFN-β-expressing cells in the PPs of IFN-β-YFP mice 48 h after infection with *Y. enterocolitica*. (E) (Top) Mice with *Y. enterocolitica* primary infection were treated with recombinant IFN-ζ during the first 3 days of reinfection (IFN-ζ treatment, $n = 6$; no-treatment control, $n = 15$; NS, not significant). (Bottom) Mice with *Y. enterocolitica* primary infected were treated with anti-IFNAR1 antibody during reinfection ($n = 7$ each; *, $P < 0.05$). (F) Real-time PCR analysis of gene expressions in the MLN taken from mice treated with anti-IFNAR1 antibody during reinfection ($n = 5$ each; *, $P < 0.05$; NS, not significant). (G) FCM analysis of IFN-γ- and IL-17-expressing CD4$^+$ T cells isolated from the MLN of mice treated with anti-IFNAR1 antibody ($n = 4$ each; data are means ± SEM).

resistance to reinfection. Since Trif^{LPS2} mice could not operate memory immunity even after compensation of IFN-ζ, multiple defects are involved in the operation of memory immunity in Trif^{LPS2} mice, not just impaired induction of type I IFNs. Therefore, regulation of Th cell differentiation during infection with Gram-negative bacteria involves TRIF-mediated multiple cytokine pathways that are integrated with type I IFN expression by APCs and STAT1 activation in T cells.

Although a majority of CD4$^+$ effector T cells die after terminal differentiation and activation, a portion of these T cells survive long enough to achieve memory function (25, 26). It has been suggested that weaker antigen presentation to T cells tends to generate T_{CM} and Th17 cells rather than other effector cell types (26, 27), which might explain why Mϕs but not DCs manifested a TRIF-deficient phenotype in Th17 cell differentiation and memory cell generation upon Gram-negative bacterial infection. This idea may also

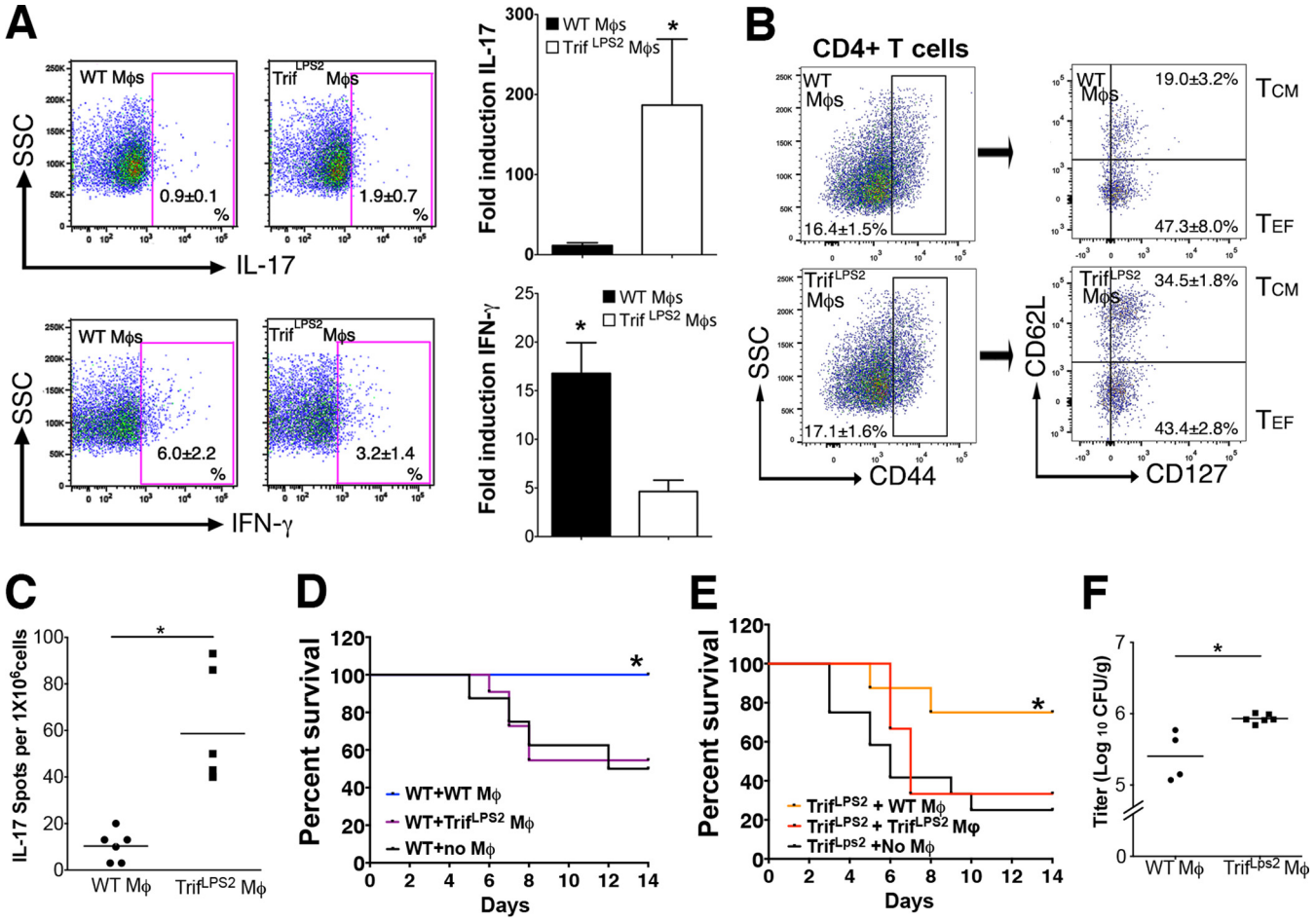

FIG 7 The role of TRIF signaling in Mϕs in establishment of Th cell-mediated memory immunity. (A and B) After 2 injections (14 days apart) of *Y. enterocolitica*-primed Mϕs, MLN CD4$^+$ T cells were examined for the expression of IL-17 and IFN-γ along with memory markers by FCM ($n = 4$ each; data are means ± SEM). The graphs show induction of IL-17 and IFN-γ mRNA in CD4$^+$ T cells from the MLN calculated based on real-time PCR data before and after Mϕ immunization. (C) ELISPOT assay showing *Y. enterocolitica*-specific CD4$^+$ Th17 cells from the MLN in the Mϕ immunization model (*, $P < 0.05$). (D and E) Survival of WT and Trif^{LPS2} mice immunized with *Y. enterocolitica*-primed Mϕs after *Y. enterocolitica* infection (5×10^7 CFU). (F) *Y. enterocolitica* colonization in the spleen 7 days postinfection in the Mϕ immunization models (*, $P < 0.05$).

apply to lineage-specific activation of memory T cells upon reinfection, because transferring WT memory CD4$^+$ T cells into Trif^{LPS2} mice but not WT mice resulted in increased generation of T_{CM} and expression of IL-17 among those cells during infection. Therefore, not only APC regulation of Th17 cell differentiation (23) but also T_{CM} cell generation and lineage-specific activation of memory Th17 cells are regulated by TRIF signaling.

Our data also suggest novel and important roles for TRIF in innate immune cells in the induction of protective immunity that are initiated by memory T cells. Memory T cells appear to induce counteractivation of innate immune cells in order to exert protective immunity. Accumulating evidence has highlighted granulopoiesis, neutrophil influx, and secretion of antimicrobial peptides as antimicrobial properties of Th17 cells (13, 28). These properties are mediated by either IL-17 or IL-22 secreted from Th17 cells and facilitate bacterial clearance (29, 30). TRIF signaling is still required for innate immune cells to receive these effects from memory cells, because Trif^{LPS2} mice could not resist secondary infection with *Y. enterocolitica* despite abundant generation of Th17 cells. Trif^{LPS2} mice could not achieve resistance to secondary infection even after transfer of memory T cells from WT mice. Although the response of Trif^{LPS2} Mϕs to IL-17 was equivalent to that of WT Mϕs in the induction of CXC chemokines *in vitro*, Trif^{LPS2} mice did not produce sufficient CXC chemokines to recruit neutrophils in the PPs during *in vivo* infection with *Y. enterocolitica*. This indicates that the expression of CXC chemokines in the PPs during infection with *Y. enterocolitica* relies predominantly on LPS-induced TLR4 signaling rather than IL-17R signaling in response to IL-17 secreted by Th17 cells (31–33). This was also true in Mϕ bactericidal function, in which Th17 cell-mediated acceleration of bactericidal function still requires TRIF signaling for full spectrum. Although Trif^{LPS2} Mϕs responded to Th17 cells, overall suppression of bacterial growth in Trif^{LPS2} Mϕs was still significantly lower than that in WT Mϕs. This may be due to a fundamental phagocytic defect in Trif^{LPS2} Mϕs, as we showed in our earlier report (7). In addition, TRIF-mediated interaction of APCs to memory T cells seems to be crucial for memory immunity to eliminate Gram-negative pathogens in the secondary infection because memory T cells require CD28 costimulation for maximal expansion (34).

Our findings suggest that the integration of innate and adaptive immunity against Gram-negative infections is regulated by TRIF for both induction and operation of immunological memory. TRIF appears to be involved in the regulation of Th17 cell differentiation from naive as well as resting memory $CD4^+$ T cells and their functions via induction of the IFN signaling cascade in the MLN, where homeostatic proliferations of memory T cells are organized (1). Because the intestine serves as the Th17 cell factory in the body (35), targeting TRIF through intestinal mucosa may have additional potential for innovative vaccine strategies and to control many diseases that involve Th17 cell pathology.

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